High-throughput determination of dissolved free amino acids in unconcentrated freshwater by ion-pairing liquid chromatography and mass spectrometry

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High-throughput determination of dissolved free amino acids in unconcentrated freshwater by ion-pairing liquid chromatography and mass spectrometry

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Abstract

We developed a procedure for the direct determination of dissolved free amino acids (DFAAs) in freshwater samples employing ion-pairing liquid chromatography and mass spectrometry. Our approach allowed accurate quantification of subnanomolar concentrations of DFAAs without prior concentration, derivatization or sample clean-up steps, achieving a throughput of three samples per hour. DFAAs were separated on a C-18 resin using tridecafluoroheptanoic acid as an ion-pairing agent controlling the overall retention. The relative standard deviation of DFAA measurements was <10% in samples from the mesotrophic Lake Zurich (Switzerland), and across concentrations of 0.5 to 500 nM. Recoveries of DFAAs ranged from 94 to 102 % within the range of 0.2 to 10 nM. The limits of quantification for individual DFAAs varied between 50 pM to 2 nM (median, 0.5 nM). The new method was employed to compare the spatial variability of DFAA concentrations in samples obtained by two devices. Epilimnetic samples of different size (ml, l) were collected at various spatial scales (cm, m, km) with a traditional 5 l Friedinger sampler and with a custom-made multi-syringe sampling apparatus. Concentrations of total DFAAs ranged from 30 to 330 nM. Alanine, serine, glutamic acid, arginine and glycine constituted 65% of the total pool, while methionine and tryptophan occurred at sub-nM concentrations only. Concentrations of individual DFAAs varied spatially over 2 orders of magnitude. Their spatial distribution was positively skewed, as characterized by rare peaks, most strongly so for glutamate, glycine and asparagine. The composition of DFAAs significantly differed at all examined spatial scales, and this could be mainly attributed to alanine, aspartic acid, and glycine. Our new method equals or outperforms existing ones in terms of sensitivity and reproducibility, while its procedural simplicity renders it superior for the high-throughput analysis of freshwater samples.

1. Introduction

Dissolved organic nitrogen (DON) is an important subset of the dissolved organic matter in aquatic systems. The majority of the DON pool consists of a mixture of ill-characterized polymeric compounds such as humic and fulvic substances that are recalcitrant to microbial degradation [e.g., 1]. Only a minor DON fraction is composed of labile identifiable low-molecular-weight substances including urea, amino acids, amines, nucleic acids and other nitrogen-bearing compounds [e.g., 2]. The amino acids can be further classified as dissolved free (DFAAs) and dissolved combined amino acids [3,4]. While the latter are amino acids combined with polypeptides, glycoproteins, humic substances or adsorbed to clay particles, DFAAs are single free amino acids readily available to microbial consumers [5,6].

Processes responsible for the release of DFAAs into the water column include phytoplankton exudation, zooplankton sloppy feeding and excretion, protistan bacterivory, viral lysis or release from detrital particles [e.g., 7]. Microbial uptake of DFAAs has been extensively studied in a variety of aquatic ecosystems [e.g., 6,8,9]. Heterotrophic bacteria are the primary consumers of DFAAs [e.g., 9,10], but other organisms such as photoheterotrophic cyanobacteria [11,12] or algae [13] may also substantially contribute to bulk DFAA uptake. At the community level, the utilization of DFAAs is proportionally related to their availability [14], whereas spectra of amino acids incorporated by different microbial populations substantially differ [15,16]. Although individual DFAAs are typically available at low (to sub-)nM concentrations only [17], the DFAA pool may satisfy a large fraction of bacterial N and C demands [6,9], which emphasizes their importance for bacterial growth [e.g., 18]. Low ambient DFAA concentrations may also be due to efficient bacterial uptake [e.g., 4]. Consequently, the release and uptake of DFAAs tend to be tightly linked [19], and individual DFAAs are characterized by short turnover times in the range of a few hours [e.g., 20,21].

Dissolved substances are not homogeneously distributed in planktonic habitats. Instead, there is a continuous formation of transient microscale substrate patches [22]. Pronounced DOM concentration gradients may be detectable even at the µm-range [23], as demonstrated for DFAAs released from organic 'lake snow' aggregates [24]. This heterogeneity is, moreover, reflected by a not random, patchy distribution of -presumably motile [22]- bacteria at the cm scale [25,26]. The concentrations of DFAAs also vary with depth, time of the day or season [27-29].

The most widely-used technique for the determination of DFAAs in aquatic systems is based on reversed-phase liquid chromatography after pre-column derivatization with *o*-phthaldialdehyde and subsequent fluorimetric detection [30]. Although this method is sufficiently sensitive to detect ambient amino acid concentrations, a complete chromatographic separation of DFAAs is required to allow for accurate quantification. However, the latter might be compromised by co-eluting compounds or matrix effects. In addition, the derivatization step is a potential source of variability. Therefore, other approaches, particularly focused on the detection, might be beneficial for facilitating amino acid determination in natural freshwaters.

Recently, methods employing reversed-phase liquid chromatography with volatile perfluorinated carboxylic acids as ion-pairing reagents [31] coupled to mass spectrometry have been applied to detect underivatized amino acids [e.g., 32] and small peptides [33] in biological fluids, albeit only at µM levels. The goal of our study was to adapt these approaches for the accurate determination of DFAAs at low nM concentrations in lake water samples. In order to illustrate the potential of the new method for high throughput analysis, we assessed the horizontal variability of DFAAs in surface waters of a large mesotrophic lake at different spatial scales and using two distinct sampling devices.

2. Material and methods

2.1. Reagents

L-alanine (Ala), L-arginine (Arg), L-asparagine (Asn), L-aspartic acid (Asp), L-cysteine (Cys), 2,6-diaminopimelic acid (Dapa), γ-Aminobutyric acid (Gaba, internal standard), L-glutamine (Gln), L-glutamic acid (Glu), L-glycine (Gly), L-histidine (His), L-hydroxyproline (Hyp), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-ornithine (Orn), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser), taurine (Tau), L-threonine (Thr), L-tryptophan (Trp), L-tyrosine (Tyr), L-valine (Val) were purchased from Sigma-Aldrich at >98% purity grade. L-leucine-[$^{13}C_6$, 99%] (Cambridge Isotope Laboratories) was used as an additional internal standard. Tridecafluoroheptanoic acid (TDFHA, 99%) and LC-MS-grade acetonitrile were obtained from Sigma-Aldrich and J.T.Baker, Avantor, respectively. Water was purified in a Milli-Q Direct 8 system (Millipore) and was used for the preparation of solvents and aqueous reagents.

2.2. Sample collection and preparation

To assess the spatial variations in the concentrations of dissolved free amino acids (DFAAs), 5 stations were sampled at a depth of 5 m on 09 April 2014 along the longitudinal axis of the large mesotrophic prealpine Lake Zurich (406 m above sea level, area 65.06 km^2, max. depth 136 m, mean depth 51.7 m, residence time 440 d, 47°17'N, 8°36'E, Switzerland) [34]. The distance between station I and stations II, III, IV, V was 4, 5.5, 12, 22 km, respectively. We used a custom-made multi-syringe sampling device that consisted of 3 arms arranged at an angle of 120°, with a distance of 1 m between the end points of neighboring arms, covering a horizontal

area of 0.43 m^2. Each arm held 3 sterile syringes (volume 20 ml, Braun) to collect samples at 1 cm distance (Fig. 1). Syringe plungers were operated pneumatically. In addition, triplicate samples were also collected with a 5 l Friedinger sampler (diameter 14 cm, sampled area 0.0154 m^2, integrated depth 33 cm, Uwitec, Mondsee, Austria). Freshly obtained samples were immediately filtered through a polyethersulfone syringe filter (pore size 0.2 µm, diameter 13 mm, Pall) and kept on ice in the dark until further processing. Prior to the filtration, the syringes and filters were washed 3 times with ultra-pure water, and the first ml of the filtrate was discarded. Filtrates of 1.5 ml were collected into HPLC vials (volume 2 ml, Glastechnik Graefenroda) and stored at -20°C until further processed. The effect of sample filtration was evaluated according to [35]. In parallel, total bacterial abundances in each sample were measured by flow cytometry as described previously [16].

2.3. Ion-pairing liquid chromatography

Analyses of DFAAs were performed with a HPLC system (1260 Infinity series, Agilent Technologies) equipped with a degasser, a binary pump, an autosampler and a column oven. Analytes were separated on an Uptisphere X-Serie C-18 column (50 × 2.1 mm, 3 µm particle size, Interchim) protected by a column pre-filter (pore size 0.45 μm, Brechbühler). The mobile phase flow rate was 500 µl min^{-1}. Solvent A was 0.5 mM tridecafluoroheptanoic acid (TDFHA) in water, and solvent B was 100% acetonitrile. Separation was accomplished using a gradient elution as follows: 0% B for 0.8 min, then 0% to 15% B from 0.8 to 1.2 min, 15% B was maintained for 2 min, then 15% to 30% B from 3.2 to 4.4 min, and 30% B was maintained for 1.2 min, then 30% to 75% B from 6 to 7.2 min, then 75% to 100% B in 0.1 min, and this was maintained for 4 min. The column was then returned to 0% B in 0.1 min and reconditioned from 11.4 to 20 min. The column was operated at 30°C and samples were kept at 4°C during analyses.

The injection volume was 10 µl. An internal standard (100 nM γ-Aminobutyric acid) was injected from an external vial 5 min after the sample injection by a custom-defined injection sequence. Prior to the first sample injection, the column was equilibrated for 20 min at 100% A until a stable back pressure level was reached, followed by 5 blank injections using the gradient elution as detailed above.

2.4. Mass spectrometry

DFAAs were detected on an API 5000 triple quadrupole mass spectrometer (AB Sciex) equipped with an electrospray ionization probe. Using a scheduled multiple reaction monitoring scan mode, DFAAs were detected in the positive ion mode. For the optimization of mass spectrometry conditions tandem mass spectrometry (MS/MS) analyses were applied to obtain compound-specific transitions (pairs of precursor and product ions) with their specific declustering potential (DP), entering potential (EP), collision energy (CE) and cell exit potential (CXP) parameters (Table 1). For all transitions EP was set to 10 V. For each amino acid, product ion mass spectra (product ion scan mode) were acquired from the precursor ion $[M+H]^+$ and the 2 most abundant or the most specific product ions were selected (only 1 transition was used for alanine and glycine). For all scans, the lower *m/z* threshold was set to 25. Further, ion-spray voltage (IS), temperature (TEM) as well as curtain (CUR), collision (CAD) and ion source gas (GS1 and GS2) parameters were optimized to obtain highest signal intensities of the selected transitions. After optimization the ion source parameters were set as follows: IS = 4500 V, TEM = 650°C, CUR = 10, CAD = 10, GS1 = 50, GS2 = 60. Nitrogen was used as curtain, collision and ion source gas. For all transitions the dwell time was set to 50 ms. Transitions will be denoted as in the following example: a molecule with a precursor ion at *m/z* 90 and a product ion at *m/z* 44 will be given as 90/44. Details on the transitions are given in Table 1. The software Analyst

158 (version 1.6.1, AB Sciex) was used for the data acquisition. Measurements of the extracted ion
159 chromatographs (XIC) for each of the monitored transitions (pairs of precursor and product ions)
160 were subsequently quantified by integration algorithms implemented in the MultiQuant software
161 (version 2.1, AB Sciex). For each amino acid, a calibration curve from 50 pM to 500 nM
162 concentrations and the corresponding response factor were obtained using γ-Aminobutyric acid
163 or ^{13}C-Leu as internal standards. Either a linear or Hill regression was applied to obtain the best
164 fit for the calibration curves. ^{13}C-Leu was discriminated from natural Leu based on differences in
165 *m/z* corresponding to the number of ^{13}C atoms. Unfortunately, Lys had be excluded from the data
166 due to incorrectly applied retention time in a subset of samples, which precluded a detection of
167 the corresponding peak and its proper quantification. Analyses of lake water samples did not
168 include Tau, Hyp, Dapa, and Orn because these amino acids were only implemented into the
169 method afterwards.
170
171 *2.5. Detection and quantification limits, method precision*
172 Limits of detection (LOD) were determined as the lowest concentration at which the
173 studied analytes yielded a signal to noise ratio of >5 [36]. Method precision, assessed as the
174 relative standard deviation, was calculated from replicate measurements (n = 6) of lake water
175 spiked with known concentrations of selected compounds. Limits of quantification (LOQ) were
176 defined as the lowest analyte concentrations with relative standard deviation <10 % [37].
177
178 *2.6. Statistical analyses*
179 Data on the composition of the DFAA pool in samples collected by the syringe sampler (n
180 = 45) were first standardized by calculating the measured concentrations of each amino acid as a
181 proportion of the total DFAA concentration. Then, a similarity matrix was constructed using the

Bray-Curtis similarity measure. The latter approach was applied because of its strength to deal with zero values in a meaningful manner. A non-metric multidimensional scaling analysis (NMDS) was used to ordinate the similarity data. Further, an analysis of similarities (ANOSIM) was applied to examine significant differences in the composition of DFAAs between the sampling sites. The average contribution of individual DFAAs to the observed dissimilarities between the compared samples was assessed by a similarity percentage analysis (SIMPER). Statistical analyses were performed using the programme Paleontological Statistics (PAST, http://folk.uio.no/ohammer/past).

3. Results

3.1. Optimization of chromatographic conditions

Volatile perfluorinated organic acids, such as e.g. tridecafluoroheptanoic acid (TDFHA), are commonly used as ion-pairing reagents in reversed-phase chromatography and are readily compatible with mass spectrometric detection [31,33]. TDFHA has been previously shown to allow for a retention and separation of amino acids on C-18 columns employing gradient elutions with acetonitrile as organic modifier [38,39]. Apart from the type of ion-pairing reagent, the overall retention of DFAAs also depends on its concentration. Therefore, gradient elutions with different TDFHA concentrations (0.05, 0.1, 0.25, 0.5 mM) were performed and evaluated. To optimize the duration and shape (linear vs. stepwise changes) of the gradient elution, concentration of acetonitrile as well as re-equilibration time between two injections, further tests with defined DFAA mixtures were performed. As expected, the retention of DFAAs increased with increasing concentration of TDFHA. We found that a stepwise acetonitrile gradient using 0.5 mM TDFHA resulted in the best retention of DFAAs. Using the proposed chromatographic conditions, twenty-four amino acids could be accurately determined within 7 min (Fig. 2).

206 Overall, the elution order followed the balance between the hydrophilic and hydrophobic
207 properties of DFAAs; the acidic and amidic amino acids were eluted first, while non-polar,
208 aromatic and basic amino acids were retained more strongly (Fig. 2). Co-eluting compounds
209 could be unambiguously identified and quantified with mass spectrometry. Detection of Dapa
210 required addition of the ion-pairing reagent (0.5 mM TDFHA) into the sample prior to the
211 analysis, but this step did not influence the determination of other amino acids. Tau eluted in the
212 void volume, as reported previously [38,39]. To prevent deterioration of chromatographic
213 conditions between individual samples, the column was flushed with 100% acetonitrile after the
214 elution was accomplished in order to remove potential contaminants. A relatively long re-
215 equilibration time of 9 min was found to be beneficial for obtaining stable and reproducible
216 retention times. No significant shifts in DFAA retention times were observed over a 1 year period
217 of intense column use.
218 For Trp, His, Lys, Orn and Arg, signal interference at 0.5 mM concentration of the ion-
219 pairing reagent were observed (data not shown), which, nevertheless, did not substantially
220 compromise the quantification due to the generally high signal intensities of the analytes. These
221 interferences were most likely related to the presence of TDFHA adducts, as described previously
222 [39]. The latter could be avoided by using lower concentration of the ion-pairing reagent (e.g. 0.1
223 mM) which, however, results in somewhat reduced retention of the analytes.
224
225 *3.2. Method precision, detection limits, compound recoveries and signal interference*
226 For most amino acids, the mean relative standard deviations (n = 6) were typically <10 %
227 over concentration ranges from 0.5 to 500 nM, while they increased to 20-40 % at concentrations
228 < 0.5 nM. The limits of detection (LOD) varied between 50 and 500 pM. The limits of
229 quantification (LOQ) ranged from 50 pM to 2 nM and were mostly at 0.5 nM (Table 1). No

230 significant differences in compound concentrations were found before and after the filtration of
231 samples through 0.2 µm PES membrane (data not shown). Recoveries of the analytes ranged
232 from 94 to 102 % at 0.2-10 nM levels. For nearly all studied analytes, no significant matrix
233 interferences were detected in samples from Lake Zurich, as compared to signals obtained with
234 amino acid standards diluted in MilliQ-water (data not shown). Minor matrix interference was
235 observed for Glu and Asp, but in both cases the background signal levels remained low.
236 Moreover, signals obtained from all transitions (pairs of precursor and product ions)
237 simultaneously monitored within one detection window that were further used for quantitative
238 analyses, were tested for the presence of interferences between individual analytes. A minor
239 interference of Asn with the signal of Asp (<6 %) was observed, but only at elevated
240 concentrations (100 nM). This interference was negligible (<1 %) at concentrations <10 nM
241 typically found in natural freshwater habitats. Isobaric Gln and Lys were chromatographically
242 completely resolved (Fig. 2, retention times of 0.8 and 6.5 min, respectively) which allowed for
243 their accurate determination in two separate detection windows using a common transition
244 (147/84). Nearly a baseline separation of isomeric Ile and Leu was achieved (Fig. 2, Suppl. Fig.
245 1) which was essential for their unambiguous detection. Ile was quantified using a specific
246 transition (132/69) yielding virtually no interference in Leu signal, whereas a non-specific
247 transition (132/86) targeting both Leu and Ile was, in turn, used for Leu quantification. In
248 addition, Leu was also determined based on a complementary transition (132/43) with negligible
249 interference in Ile signal (Suppl. Fig. 1).
250
251 *3.3. Variations in total DFAAs and bacteria at different sampling scales*
252 Average concentrations of the total dissolved free amino acids (DFAAs) ranged from 71
253 to 196 nM, and from 17 to 106 nM in the samples collected by the syringe and the Friedinger

sampler, respectively (Fig. 3a). Apart from sampling station II, the average total DFAA concentrations were not significantly different between samples collected by the two sampling devices (Fig. 3a). However, coefficients of variation varied between 20 and 120 % in the samples from the syringe sampler, which was approximately 3 to 25-times higher (except for the station V) than in samples obtained by the Friedinger sampler. Total bacterial abundances also varied between different sampling stations in the range of $0.8 – 3.4 \times 10^6$ cells ml^{-1} (Suppl. Fig. 2). Moreover, samples collected by the different samplers or at different arms of the syringe sampler yielded significant differences in average bacterial abundances.

The degree of variability in the total DFAA concentrations was even more pronounced between the triplicate samples of 20 mL collected at a distance of 1 m from each other (Fig. 3b). Although the differences in the average total DFAA concentrations were mostly insignificant, the sets of samples were nevertheless characterized by distinctly different ranges of values. The corresponding coefficients of variation of the total DFAA concentrations ranged from 7 to 104 %.

3.4. Concentrations of individual DFAAs

The concentrations of all amino acids in the samples collected by the syringe sampler (n = 45) typically varied over 2 orders of magnitude, with the mean concentrations substantially higher than the median ones (Fig. 4a). Median concentrations of DFAAs were in the low nM range except for Trp and Met that occurred at sub-nM concentrations only (Fig. 4a). Ala and Ser were quantitatively the most important components with median concentrations of approximately 10 nM. The skewness of the concentration distributions of all DFAAs was >0.5, i.e., substantially deviated from normality (Fig. 4b). All DFAAs were thus characterized by the rare occurrence of elevated concentrations, albeit to a different degree. For example -even though the median concentrations of the two DFAAs were comparable- Glu appeared to be distributed in a very

patchy manner (Fig. 4c), whereas the distribution of Arg concentrations was only slightly skewed.

3.5. Changes of the DFAA pool at different spatial scales

Ala, Ser, Glu, Asp and Arg quantitatively dominated in samples collected by the syringe sampler (n = 45) and constituted on average 65 % of the total DFAA pool (Fig. 5a). In contrast, Thr, Leu, Met, aromatic and amidic amino acids accounted each for < 3 % of the total pool. No significant differences in the relative composition of the DFAA pool were found between sampling stations I, II, and III (Table 2). In contrast, the composition of DFAAs at station IV was significantly different from the previous 3 stations and that at station V significantly differed from stations I and II, respectively (Table 2, ANOSIM analysis, n = 45). The overall average dissimilarity in the relative DFAA composition between the sampling stations was 36 %. This was mainly due to Ala, Asp, and Gly, which together accounted for 45 % of the overall dissimilarity (SIMPER analysis, n = 45). Pair-wise comparisons of the sampling stations further indicated that the overall average dissimilarity in the relative composition of the DFAA pool increased from 23 to 42 % with increasing distance between the sites; most notably between stations III, IV and V. Ordination analysis (non-metric multidimensional scaling) additionally revealed significantly different DFAA composition between replicate samples collected 1 cm apart from each other, as found for instance at station V (Fig. 5b). On the other hand, a greater difference in the composition of the DFAA pool was found at station III between triplicates sampled at a distance of 1 m than between samples collected at a spatial scale of 1 cm (Fig. 5b). In addition, the composition of DFAAs at stations II and IV significantly differed between samples collected by the Friedinger and syringe samplers (data not shown).

4. Discussion

4.1. Detection of DFAAs

In total, a variety of DFAAs could be directly detected in aquatic samples from Lake Zurich after a simple filtration step by the optimized method based on ion-pairing liquid-chromatography coupled to mass spectrometry. Our approach allowed for a direct and simultaneous detection of DFAAs even at sub-nM concentrations, which is comparable to the threshold concentrations reported for other approaches [32]. Apart from high sensitivity, the major advantage of the method lies in its excellent specificity. Although we did not achieve as good separation of hydrophilic amino acids as the classic derivatization method [30], mass spectrometric detection permitted their unambiguous quantification. We could thus accurately measure the ambient concentrations of DFAAs in lake water samples with reduced preparative effort and consequently higher throughput compared to protocols requiring prior derivatization [30]. In addition, our technique also allows for a quantification of Pro, which, bearing a secondary amine, resists detection by the commonly applied derivatization method with *o*-phthaldialdehyde [30].

4.2. Spatial variability

Epilimnetic samples of 20 ml collected from Lake Zurich by the syringe sampler at distances of 1 cm clearly showed that the ambient concentrations of DFAAs were highly variable at this scale (Figs. 3b, 5b). These variations in total DFAA concentrations were higher by 3 to 25 times in the 20 ml syringe samples than in replicate samples of 5 l from the Friedinger sampler (Fig. 3a). The higher variability at the smaller sampling scale demonstrated the existence of pronounced heterogeneity in DFAAs. Our data thus agree with the widespread conceptual understanding that natural aquatic ecosystems are spatially heterogeneous [40,41] and, to the best

of our knowledge, provide first field evidence for such extremely patchy distribution of DFAAs at the scale of cm to m in a lake. Heterogeneous distributions of individual DFAAs at small sampling and spatial scales were mainly due to locally elevated concentrations resulting in their positively skewed distributions (Fig. 4). This implies that the ambient DFAA pool in freshwater that is most frequently experienced by microbes is usually at the lower range of the measured concentrations, whereas patches of increased concentrations only occur sporadically. It is unlikely that the significant differences in the DFAA composition were due to sampling artefacts since these differences were randomly distributed across different arms and syringes of the sampler, and syringes had been thoroughly cleaned before each use to avoid cross-contamination. It seems unlikely that the higher variability in DFAA concentrations in syringe samples was a result of sampling procedure, i.e, that the pressure change during sampling might have disrupted some delicate cells (e.g. ciliates): subsamples for DFAA analyses from the Friedinger sampler were also taken with a syringe and subsequently filtered through 0.2 µm membrane. Therefore, any potential sampling artefact is likely comparable between both types of samples. Further, the precision of the method was much higher than the observed variability, i.e., < 10 % at 0.5 – 10 nM levels in triplicate measurements of lake water spiked with a defined mixture of DFAAs. This was substantially less than the differences between the syringe samples at the scale of 1 cm or 1 m. It is conceivable that individual samples of 20 ml were taken from volumes of water randomly containing point sources of amino acids. Such pronounced heterogeneity could not be effectively inferred from the larger sized samples (5 l), likely because a thorough mixing of water masses during sampling is disruptive to fragile organic particles and substrate gradients in the near vicinity of individual phytoplankton cells, zooplankton, lysed cells, or organic particles [42-45]. Assuming that the differences in the composition of the DFAA pool were directly related to the sample size, a volume of 1 l seemed to be more representative of the median DFAA

concentrations of the spatial variability, reflecting the larger *in situ* dynamics. By contrast, samples of 20 ml were more appropriate for apprehending the actual heterogeneity of their local distribution.

4.3. Composition of the DFAA pool

The average composition of the DFAA pool in the epilimnion of Lake Zurich (Fig. 5a) was generally consistent with previous studies from diverse planktonic habitats [27,46] that typically recognized Ala, Ser, and Gly among the quantitatively most prominent DFAAs. In contrast, Trp and Met were usually reported as rare components of the DFAA pool. Other DFAAs occurred at intermediate proportions. The quantitative dominance of Ala, Ser, and Gly might be explained by their low bacterial uptake [16], high phytoplankton release [14] or by protein degradation [47]. Nevertheless, depending on the type of a sampled system, the composition of the DFAA pool substantially varied, and could even be entirely dominated by amino acids that are usually rare in pelagic habitats (such as Asn), as e.g., determined in drainage samples from a boreal catchment [48]. Overall, the range of total DFAA concentrations detected in this study are in agreement with concentrations typically reported for freshwater lakes [49]. They tend to be at the lower range of values, compared to the only previous measurements conducted in Lake Zurich [50], which might be a consequence of the re-oligotrophication of the lake during the past decades [51].

Despite the overall apparent uniformity in the average composition of DFAAs, there were significant differences between the sampling stations (Table 2) as well as at the scales of 1 m and even of 1 cm (Fig. 5b). Ala quantitatively dominated the DFAA pool with the most pronounced differences in the average proportion between the sampling sites and contributed most to the overall dissimilarity. Significant parts of the overall heterogeneity in the DFAA composition

374 could be further explained by variations in the concentrations of Asp and Gly, while the
375 contributions of all other DFAAs were insignificant. Since the compositional data were based on
376 the absolute concentrations of DFAAs, it was expected that the differences within the pool would
377 be driven by its dominant components. In contrast, our data indicated that Asp and Gly
378 contributed disproportionally more to the overall dissimilarity despite their intermediate pool
379 sizes (Fig. 4a). While the processes generating such heterogeneity remain uninvestigated, we
380 speculate that the observed variations in concentrations might be related to the patchy spatial
381 distribution of the dominant primary producer in Lake Zurich, *Planktothrix rubescens* [52]. In
382 contrast to planktonic bacteria, these cyanobacteria have been shown to preferentially incorporate
383 Asp and Gly [16].
384
385 **5. Conclusions and recommendations**
386 The here described procedure is sufficiently sensitive to directly determine DFAAs in
387 very small sample volumes (10 µl). Apart from proteinogenic amino acids, other ones that are
388 commonly found in aquatic systems, such as e.g. ornithine, diaminopimelic acid, hydroxyproline,
389 or taurine can be quantified. Moreover, the method could be easily adapted for the analysis of
390 other small peptides without substantial changes in chromatographic conditions. On the other
391 hand, amino acid enantiomers cannot be distinguished by this technique. The detection of
392 DFAAs in Lake Zurich was not compromised by interferences due to sample matrix, suggesting
393 comparable detection limits also in samples from other freshwater habitats. To test for the
394 potential matrix effects -as manifested, for instance, by unknown peaks or increased background
395 levels- in samples with different matrix complexity than Lake Zurich, stable isotope-labeled
396 amino acid analogs could be used. These are also recommended as internal standards due to their
397 identical retention times as their natural counterparts. It is advisable to detect individual amino

acids only at their expected retention times using time-specific detection windows (scheduled MRM detection mode) rather than to simultaneously monitor all throughout the whole run (standard MRM detection mode). This will allow to shorten the cycle time that is needed to properly characterize signals (i.e. to maximize the number of data points across a peak), particularly close to the detection limit. The latter may also reduce the risk of potential signal interferences between different analytes. The method yields a stable and reproducible chromatographic separation of the tested DFAAs and allows for processing of a large number of samples without operator`s actions. Nevertheless, it is advisable to periodically check their actual retention times. While minor shifts (< 0.5 min) may sometimes occur, they can be readily corrected for by the internal standard and thus do not negatively affect analytical precision. However, it is recommended to thoroughly flush the column with acetonitrile, should such shifts markedly increase or if deteriorated peak shapes or loss of retention are observed. The proposed method has been optimized for a high throughput analysis. If required, the separation of co-eluting analytes could be further improved by using lower flow rates (e.g. 200 – 300 µl min^{-1}) and/or larger columns (e.g. 100 × 2.1 mm). Using a novel multi-syringe sampling device, we obtained additional insight into spatial heterogeneity of DFAAs in the epilimnion of a large lake that could not be inferred from the samples collected by a common limnological sampling device. Therefore, sample size and scale should be carefully considered to better apprehend the heterogeneous *in situ* distribution of DFAAs.

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561 Short-term displacement of *Planktothrix rubescens* (cyanobacteria) in a pre-alpine lake
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Figure captions:

Fig. 1. Top view (scheme) of the multi-syringe sampling device. The sampler has three arms (A, B, C) arranged at an angle of 120°, with a distance of 1 m between the endpoints of the arms. Each arm holds 3 syringes (20 ml) in a horizontal distance of 1 cm. Syringe plungers are operated pneumatically. The sampler is lowered on a steel cable fixed in the center. Syringes take triplicate water samples in one depth, covering a horizontal area of 0.43 m^2 at three corners.

Fig. 2. LC-MS extracted ion chromatograms from a defined mixture of DFAAs (10 nM each) that were acquired using the proposed gradient elution with 0.5 mM TDFHA. Peaks are displayed to the normalized signal intensity (%). Ala – alanine, Arg – arginine, Asn – asparagine, Asp – aspartic acid, Cys – cysteine, Dapa – 2,6-diaminopimelic acid, Gln – glutamine, Glu – glutamic acid, Gly – glycine, His – histidine, Hyp – hydroxyproline, Ile – isoleucine, Leu – leucine, Lys – lysine, Met – methionine, Orn – ornithine, Phe – phenylalanine, Pro – proline, Ser – serine, Tau – taurine, Thr – threonine, Trp – tryptophan, Tyr – tyrosine, Val – valine. To facilitate readability, peaks are shown in separate panels. Note different scale of the *x*-axis.

Fig. 3. (A) Concentrations of total dissolved free amino acids (DFAAs) in the samples collected by the syringe and Friedinger samplers. Asterisks indicate significant differences (*t*-test, $*p < 0.05$, $**p < 0.01$) in total DFAA concentrations among the two sampling devices. Data are based on 3 replicates (for the syringe sampler, means of 3 syringes at each arm were used). (B) Concentrations of total DFAAs in the syringe samples obtained from different arms (*A, B, C*) of the sampler. Different lower case letters indicate significant differences (One-way ANOVA,

586 Tukey test, $p < 0.05$) in total DFAA concentrations between samples collected at the spatial scale
587 of 1 m. Data are based on 3 replicates. The line within the box marks the median.
588
589
590 **Fig. 4.** (A) Concentrations of individual DFAAs in epilimnetic samples collected by the syringe
591 sampler from Lake Zurich on Apr 09, 2014. The solid and bold lines within the box mark the
592 median and mean, respectively. Boundaries of the box indicate the 25^{th} and 75^{th} percentiles. Error
593 bars indicate the 10^{th} and 90^{th} percentiles. Dots represent the 5^{th} and 95^{th} percentiles ($n = 45$).
594 Arrows indicate Glu and Arg. (B) Skewness of individual DFAAs in the same data set and (C)
595 distributions of Glu and Arg *in situ* concentrations ($n = 45$). Glu (striped bars) and Arg (solid
596 black bars) are highlighted.
597
598 **Fig. 5.** (A) Relative (%) mean molar proportions of DFAAs in samples collected by the syringe
599 sampler ($n = 45$, error bars show standard errors of the mean) from Lake Zurich on Apr 09, 2014
600 and (B) non-metric multidimensional scaling plot based on Bray-Curtis similarities of the relative
601 molar composition of the total DFAA pool in the same data set. Convex hulls enclose all samples
602 of the same sampling station. The stress value of the plot is 0.162 indicating a good 2-
603 dimensional depiction of the ordination. Samples with more similar composition of DFAAs are
604 ordinated closer together. As one example of the variable DFAA pool we show samples taken at
605 the scale of 1 cm with heterogeneous and homogeneous composition of DFAAs indicated by
606 asterisks and arrows, respectively.

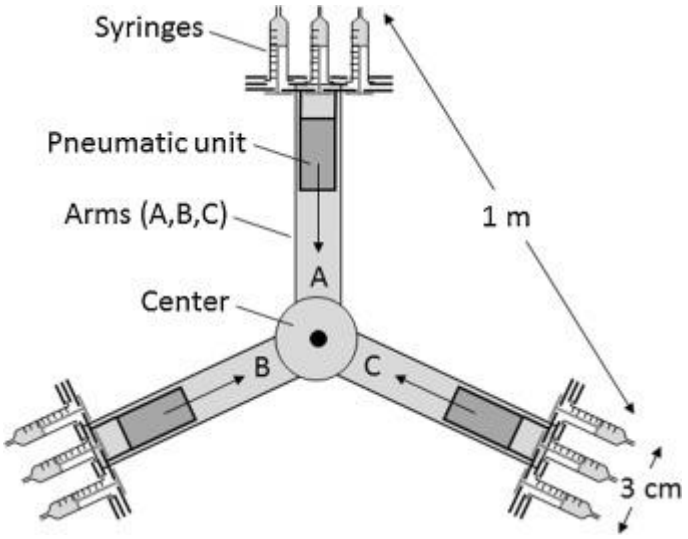

Fig.1

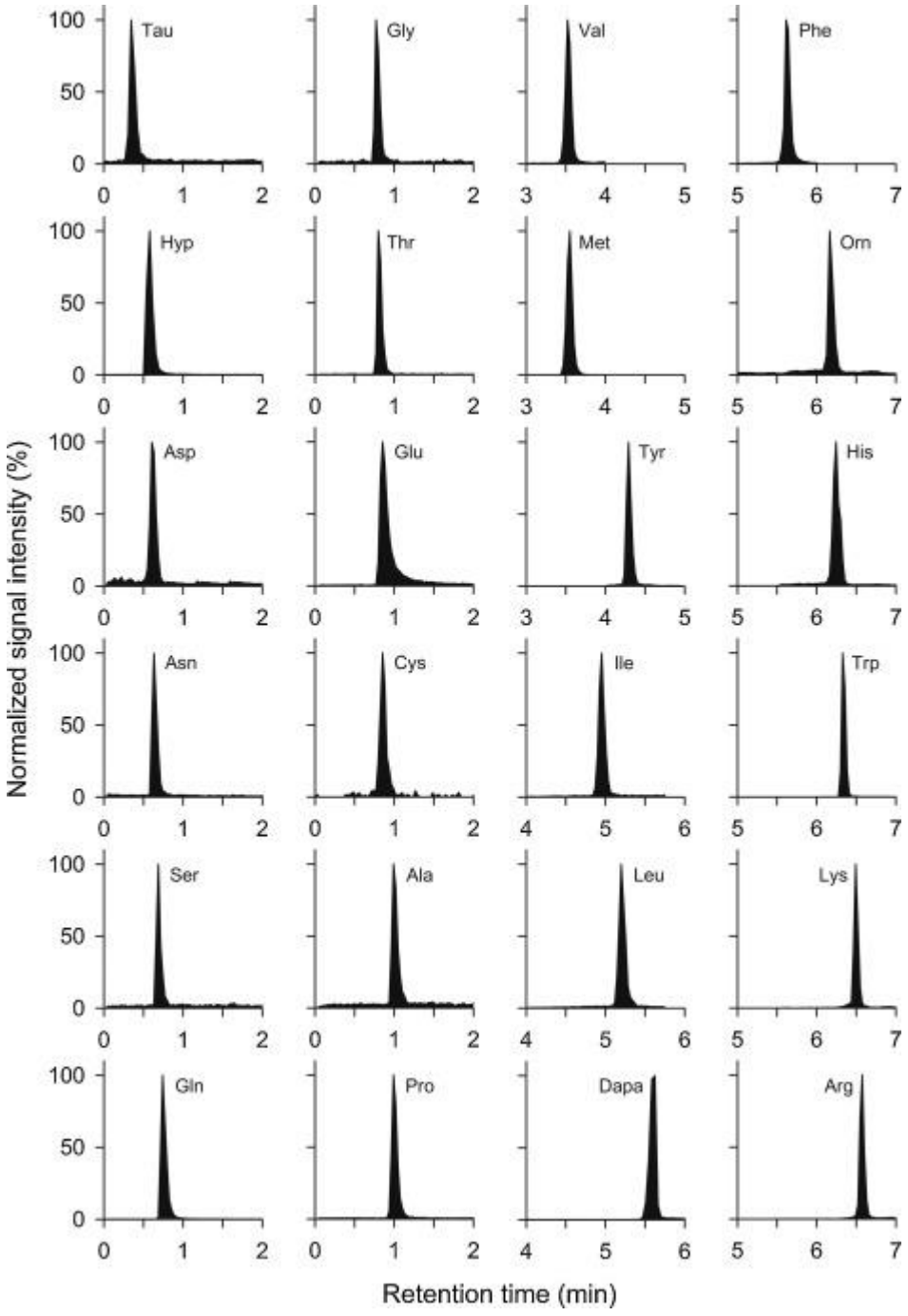
Tau
Gly
Val
Phe
Hyp
Thr
Met
Orn
Asp
Glu
Tyr
His
Asn
Cys
Ile
Trp
Ser
Ala
Leu
Lys
Gln
Pro
Dapa
Arg
Normalized signal intensity (%)
Retention time (min)

Fig. 2

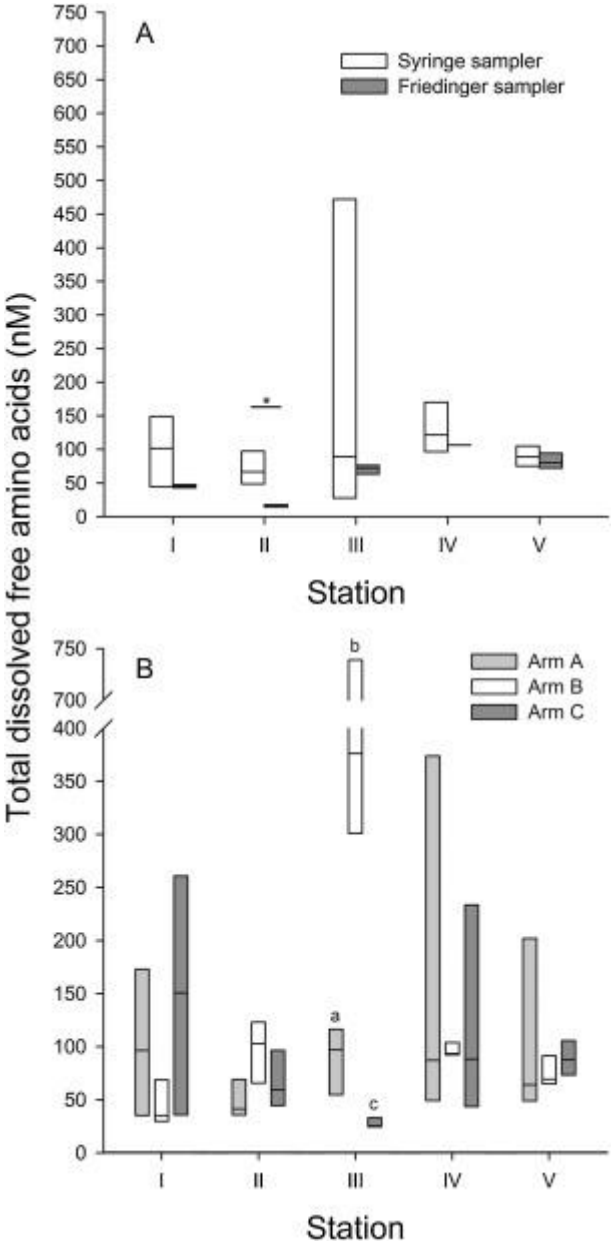
A
Syringe sampler
Friedinger sampler
Total dissolved free amino acids (nM)
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
*
I
II
III
IV
V
Station
B
Arm A
Arm B
Arm C
b
a
c
Station

Fig. 3

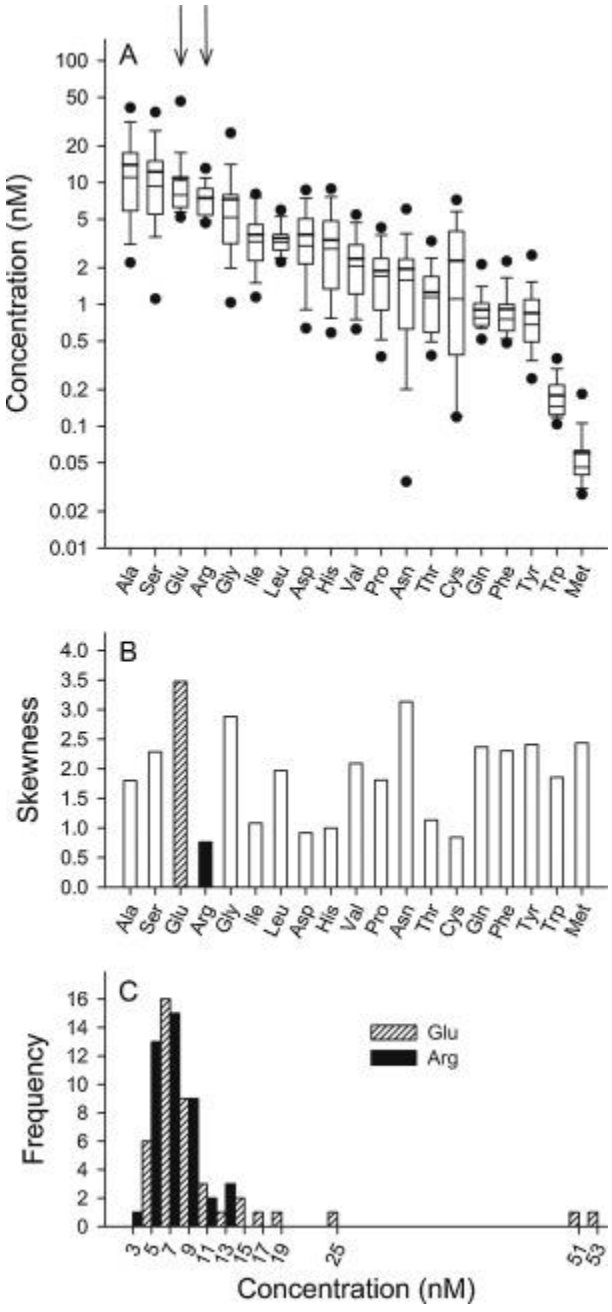
A
Concentration (nM)
100
50
20
10
5
2
1
0.5
0.2
0.1
0.05
0.02
0.01
Ala Ser Glu Arg Gly Ile Leu Asp His Val Pro Asn Thr Cys Gln Phe Tyr Trp Met
B
Skewness
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ala Ser Glu Arg Gly Ile Leu Asp His Val Pro Asn Thr Cys Gln Phe Tyr Trp Met
C
Frequency
16
14
12
10
8
6
4
2
0
Glu
Arg
3 5 7 9 11 13 15 17 19 25 51 53
Concentration (nM)

Fig. 4

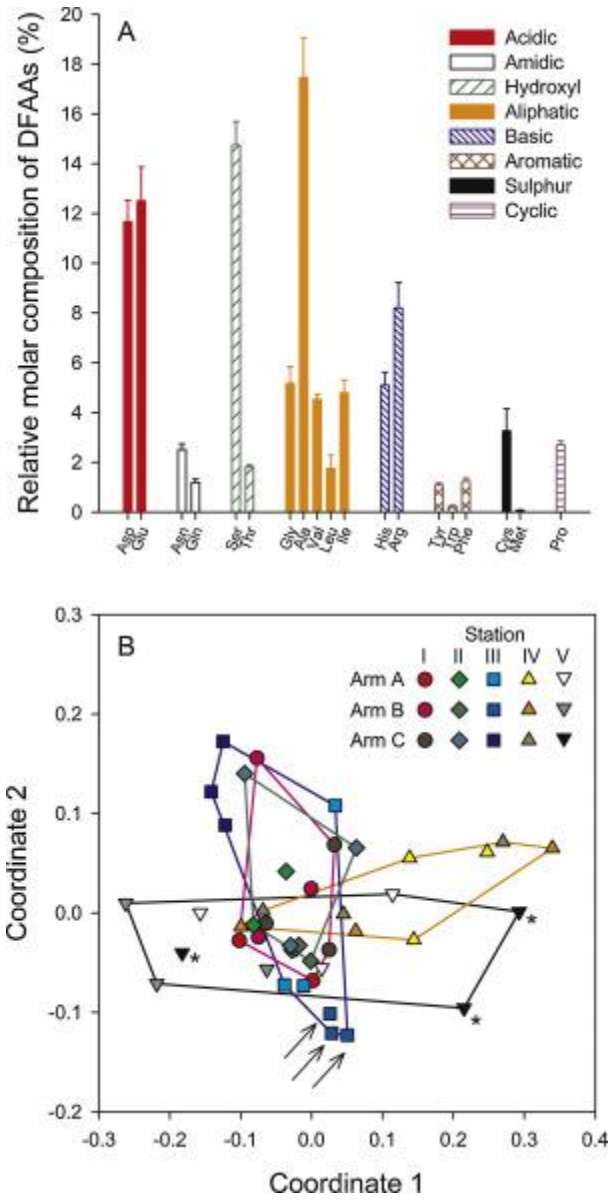
A
Relative molar composition of DFAAs (%)
Acidic
Amidic
Hydroxyl
Aliphatic
Basic
Aromatic
Sulphur
Cyclic
Asp Glu
Asn Gln
Ser Thr
Gly Ala Val Leu Ile
His Arg
Tyr Trp Phe
Cys Met
Pro
B
Station
I II III IV V
Arm A
Arm B
Arm C
Coordinate 2
Coordinate 1

Fig. 5

Table 1: Scheduled multiple reaction monitoring (sMRM) parameters applied to detect amino acids. Transitions (pairs of precursor and product ions) and the corresponding declustering potential (DP), collision energy (CE), cell exit potential (CXP) parameters, retention times (RT), limits of detection (LOD) and limits of quantification (LOQ) are shown. Ala – alanine, Arg – arginine, Asn – asparagine, Asp – aspartic acid, Cys – cysteine, Dapa – 2,6-diaminopimelic acid, Gaba – γ-Aminobutyric acid, Gln – glutamine, Glu – glutamic acid, Gly – glycine, His – histidine, Hyp – hydroxyproline, Ile – isoleucine, Leu – leucine, Lys – lysine, Met – methionine, Orn – ornithine, Phe – phenylalanine, Pro – proline, Ser – serine, Tau – taurine, Thr – threonine, Trp – tryptophan, Tyr – tyrosine, Val – valine. Transitions used for quantification are highlighted in **bold**. *sample amended with TDFHA (0.5 mM final concentration) prior to the analysis, **injection 5 min after the sample, nd – not determined

Compound	DP (V)	Transitions (m/z)	CE (V)	CXP (V)	RT (min)	LOD (nM)	LOQ (nM)
Ala	31	**90/44**	15	6	1.0	0.2	0.5
Arg	18	**175/70**	28	8	6.5	0.05	0.5
		175/116	19	6			
Asn	41	**133/87**	13	19	0.6	0.5	0.5
		133/74	21	8			
Asp	105	**134/88**	14	20	0.6	0.2	1
		134/74	19	16			
Cys	123	**122/59**	35	21	0.9	0.2	1
		122/87	15	24			
Dapa	60	**191/128**	21	20	5.7*	0.2	0.5
		191/82	35	15			
Gaba	50	**104/69**	24	18	6.7**	nd	nd
		104/59	22	7			
Gln	50	**147/84**	28	19	0.7	0.2	0.5
		147/41	40	16			

Glu	43	**148/84**	25	16	0.9	0.5	1
		148/102	18	14			
Gly	50	**76/30**	20	14	0.8	0.5	2
His	45	**156/110**	21	12	6.2	0.2	0.5
		156/83	35	8			
Hyp	60	**132/86**	20	20	0.6	0.2	0.5
		132/68	30	17			
Ile	35	**132/69**	24	30	4.9	0.2	0.5
		132/86	16	11			
Leu	35	**132/86**	16	11	5.2	0.2	0.5
		132/43	41	18			
Lys	80	**147/84**	11	14	6.5	0.2	0.5
		147/56	91	13			
Met	86	**150/56**	22	25	3.6	0.05	0.05
		150/61	34	12			
Orn	40	**133/70**	28	15	6.1	0.2	0.5
		133/116	15	20			
Phe	40	**166/120**	17	7	5.6	0.1	0.5
		166/103	24	35			
Pro	40	**116/70**	22	14	1.0	0.2	0.5
		116/45	54	8			
Ser	70	**106/60**	20	19	0.7	0.2	0.5
		106/42	29	8			
Tau	60	**126/44**	25	17	0.4	2	5
		126/108	14	25			
Thr	75	**120/74**	15	8	0.8	0.5	1
		120/56	21	14			
Trp	29	**205/146**	19	31	6.3	0.05	0.1
		205/117	31	18			
Tyr	40	**182/136**	14	28	4.3	0.1	0.2
		182/65	37	9			
Val	51	**118/72**	16	10	3.5	0.2	0.5
		118/55	25	7			

Table 2: Analysis of similarity (ANOSIM) of the DFAA composition at the five sampling stations (R statistic is shown). Similarity was calculated based on the relative composition of the DFAA pool in samples collected by the syringe sampler. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significant pair-wise differences are in **bold**, ns = not significant, $n = 9$ per station.

	Station II	Station III	Station IV	Station V
Station I	ns	ns	**0.42*** **	**0.28** **
Station II		ns	**0.35** **	**0.24** **
Station III			**0.23***	ns
Station IV				ns